Preface

Developmental biology is one of the most exciting and fast-growing fields today. In part, this is so because the subject matter deals with the innately fascinating biological events—changes in form, structure, and function of the organism. The other reason for much of the excitement in developmental biology is that the field has truly become the unifying melting pot of biology, and provides a framework that integrates anatomy, physiology, genetics, biochemistry, and cellular and molecular biology, as well as evolutionary biology. No longer is the study of embryonic development merely "embryology." In fact, development biology has produced important paradigms for both basic and clinical biomedical sciences.

Though modern developmental biology has its roots in "experimental embryology" and the even more classical "chemical embryology," the recent explosive and remarkable advances in developmental biology are critically linked to the advent of the "cellular and molecular biology revolution." The impressive arsenal of experimental and analytical tools derived from cell and molecular biology, which promise to continue to expand, together with the exponentially developing sophistication in functional imaging and information technologies, guarantee that the study of the developing embryo will contribute one of the most captivating areas of biological research in the next millennium.

There is a demonstrated need for students of developmental biology to be knowledgeable of the breadth and depth of the available experimental methodologies, by necessity derived from multiple disciplines, which are applicable to the study of the developing embryo. In particular, because developmental biology deals with multiple model systems, from organismal to tissue and cell levels, as well as a wide range of "change"-related biological activities, the investigator is often frustrated as to how his/her findings relate to those obtained in another model system and/or by using different reagents or functional markers. Compared to other more strictly defined fields of biological research, the number of "reference" publications that deal specifically with the practical aspects of experimental developmental biology are, however, relatively scarce.

Developmental Biology Protocols grows out of the need for a comprehensive laboratory manual that provides the readers the principles, background, rationale, as well as the practical protocols, for studying and analyzing the events of embryonic development. This three-volume set, consisting of 142 chapters, is intentionally broad in scope, because of the nature of modern developmental biology. Information is grouped into the following topics: (1) systems—production, culture, and storage; (2) developmental pattern and morphogenesis; (3) embryo structure and function; (4) cell lineage analysis; (5) chimeras; (6) experimental manipulation of embryos; (7) application of viral vectors; (8) organogenesis; (9) abnormal development and teratology; (10) screening and mapping of novel genes and mutations; (11) transgenesis production and gene knockout; (12) manipulation of developmental gene expression and func-

tion; (13) analysis of gene expression; (14) models of morphogenesis and development; and (15) in vitro models and analysis of differentiation and development.

Throughout *Developmental Biology Protocols*, the authors have consistently striven for a balanced presentation of both background information and actual laboratory details. It is believed that this highly practical format will permit readers to bring the concepts and principles we present into their personal research practices in a most efficient manner. Specifically, the wide range of model systems and multidisciplinary experimental techniques presented here should lower the "activation energy" for the student of developmental biology to become a contributing member of this exciting scientific discipline. In addition, teachers of developmental biology at all levels should also readily find relevant and useful information to enrich the experience of their students.

The practice of developmental biology is currently in a state of constant change, reflecting the close relationship of the field to other rapidly developing fields of biological research, particularly cell and molecular biology, and imaging and information technology. The materials presented in this three-volume set are therefore the beginning of a project that will involve continuous update and upgrade to reach and enhance the scientific endeavors of developmental biologists at large.

The production of *Developmental Biology Protocols* would not have been possible without the outstanding work of the contributing authors who share here with the readers the hands-on wisdom they have earned in the laboratory. We are grateful for their intellectual contributions as well as their remarkable tolerance to our constant reminders. Tom Lanigan and his staff at Humana Press worked diligently on the project to ensure a final product of the highest quality. Chuck, our young son, persevered throughout the gestation period of the project, and constantly demonstrated to us the meaning of "developmental biology."

Our heartfelt thanks go to Lynn Stierle, who expertly and single-handedly maintained the massive organization of the manuscripts and the correspondence (snail-mail and e-mail), as well as the sanity of the editors! Michelle Levinski also provided valuable assistance in proofreading the final production.

Finally, we hope that these volumes will find their place on the laboratory shelves, with their pages well soiled and their contents tried and tested, and prove their utility as an everyday resource for the students of developmental biology, the most exciting discipline of biology for many decades to come!

Rocky S. Tuan, PhD
Cecilia W. Lo, PhD

2

Ectopic Expression in *Drosophila*

Elizabeth L. Wilder

1. Introduction

Ectopic expression in *Drosophila* has been used extensively to examine the capabilities of a given gene in virtually any tissue. Three general approaches are described here, and the choice of which to use is determined by the needs of the particular experiment. Certain aspects of each approach can also be combined, providing powerful tools for the examination of gene function. Because ectopic expression does not involve a protocol, but rather generation of certain types of transgenic strains, this chapter focuses on a description of the approaches and in what circumstances each is likely to be useful.

2. Materials

For each of the methods of ectopic expression described here, the production of transgenic strains is required. The vectors that are widely used in these experiments are available *(1–3)*.

3. Methods
3.1. Expression Through Defined Promoters

The simplest means of ectopic expression is through the construction of a promoter-cDNA fusion in which a gene of interest is driven by a defined promoter or enhancer. Transgenic strains carrying this construct then ectopically express the gene of interest in the defined pattern.

One of the most commonly used promoters for this purpose is the heat shock protein 70 (hsp70) promoter *(1)*. This promoter allows ubiquitous expression to be induced in any tissue of the fly through a simple heat shock at 37°C. The inducible nature of this approach is a great advantage. However, basal levels of expression can be problematic, and heat shock itself can induce developmental defects. In addition, short bursts of ectopic expression ubiquitously is often not ideal. Therefore, sustained expression in defined domains may be preferred.

To achieve ectopic expression within a defined domain, transcriptional regulatory regions from characterized genes have been linked to genes of interest *(4,5)*. The advantage of this approach is its simplicity. Its primary limitation is that lethality can result from the ectopic expression. This makes it impossible to establish stable transgenic
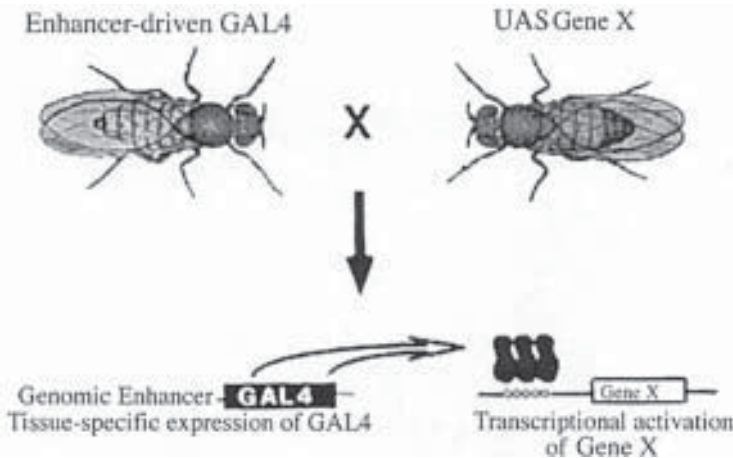
Fig. 1. The GAL4 system of ectopic expression (modified from **ref. *2***). This system allows the ectopic expression of any gene of interest (Gene X) in a pattern determined by the expression of the transcriptional activator, GAL4. Hundreds of lines in which GAL4 is expressed in a variety of patterns have been generated through enhancer trapping or by linking the GAL4 coding sequence to defined regulatory elements. These are crossed to flies carrying the gene of interest under the transcriptional control of the GAL4 Upstream Activating Sequence (UAS). The progeny of this cross express the gene of interest in the pattern of choice.

lines. Enhancers that drive expression during late stages of development or in tissues that are nonessential have been particularly useful, because lethality owing to ectopic expression is avoided.

The lethality associated with sustained expression of transgenes during development, the effort required to generate transgenic strains in which the transgene is expressed in multiple patterns, and the lack of defined enhancers driving expression in certain tissues prompted the development of alternative strategies for ectopic expression.

3.2. The GAL4 System

The identification of the yeast transcriptional activator, GAL4, as a highly active, specific transcription factor that can activate transcription in *Drosophila (6)* led to the development of a system of ectopic expression referred to as the GAL4 system *(2)*. This two-part system is shown in **Fig. 1** and involves a cross between a fly expressing GAL4 in particular cells and a fly carrying a gene of interest under the transcriptional control of the GAL4 upstream activating sequence, or UAS. In the progeny of such a cross, the gene of interest will be expressed in cells where GAL4 is synthesized. Targeted ectopic expression of the gene of interest can therefore be achieved by choosing among many strains that express GAL4 in defined patterns.

Three vectors are generally useful for investigators using this system *(2)*. pGaTB/N provides either a *Bam*HI site or a *Not*I site upstream of GAL4, allowing a defined promoter to drive GAL4 expression. The second, pGawB, is an enhancer-trapping vector that directs GAL4 expression from genomic enhancers. Finally, pUAST includes multiple cloning sites behind five copies of an ideal GAL4 binding sequence. Genes of interest are easily cloned into this vector for GAL4-mediated expression.

Hundreds of GAL4 strains have been generated through the process of enhancer trapping. These strains have been characterized by crossing newly generated lines to a UAS-LacZ strain and characterizing the expression pattern. Many of these strains are now available through the Drosophila Stock Center at Bloomington, IN. The expression patterns that have been detected through these enhancers vary from very broad expression to highly specific patterns. They, thus, offer the possibility of driving ectopic expression in virtually any tissue.

In addition to the strains generated through enhancer trapping, many lines have been generated by fusing the GAL4 coding sequence to defined promoters, such as the hsp70 promoter. The latter offers the advantage mentioned above of inducible expression. The construction of strains expressing GAL4 in defined domains allows any UAS transgene to be examined within the particular region of interest.

The GAL4 system has contributed to the utility of the FLP-FRT system of inducing mutant clones (*see* Chapter 3) *(7)*. In this system, mitotic recombination is induced via flip recombinase (FLP), which is under the control of a heat shock promoter. The resulting mutant clones are then generated in all mitotically active cell populations. However, if FLP is placed under the control of GAL4-UAS, mutant clones are only generated within the GAL4 expression domain. This allows the investigator to determine whether a particular gene has an endogenous function within cells defined by GAL4 expression.

The GAL4 system addresses many of the problems associated with simple transgenes. First, since the UAS transgenic lines are produced in the absence of GAL4 activity, ectopic expression of the transgene does not occur. Therefore, lethality associated with ectopic expression is avoided until the transgenic flies are crossed to a GAL4 expressing strain. Second, defined enhancers are not required for expression in a particular set of cells. Sites of expression are only limited by the number of enhancer trapped strains available, the number of which is continually growing. Finally, the GAL4 system allows ectopic expression in any number of patterns and conditions with the construction of only a single UAS transgene.

This system of ectopic expression is extremely powerful for these reasons, but it does have limitations. First, for undefined reasons, GAL4 does not seem to function in the germline (A. Brand, personal communication). For experiments where germline expression is needed, other methods must be used. A more universal limitation of the GAL4 system is the fact that it is not inducible. Many enhancers drive expression during early phases of development, so that GAL4-mediated ectopic expression of certain UAS transgenes results in embryonic lethality. For investigators interested in later aspects of development, this has been a serious limitation of the GAL4 system.

This problem can be partially addressed through modulation of temperature. The optimal temperature for GAL4 activity appears to be the ambient temperature for yeast, which is 30°C. By rearing flies at lower temperature, GAL4 activity is reduced *(8,9)*, and in some instances, early lethality associated with higher levels of ectopic expression from the UAS transgene is avoided. The flies can be shifted at later stages of development to increase GAL4-mediated expression.

In at least one instance, an inductive ability has been added to the GAL4 system through the construction of a UAS transgene carrying a cDNA encoding a temperature sensitive protein *(9)*. Thus, progeny of the GAL4-UAS cross are maintained at the
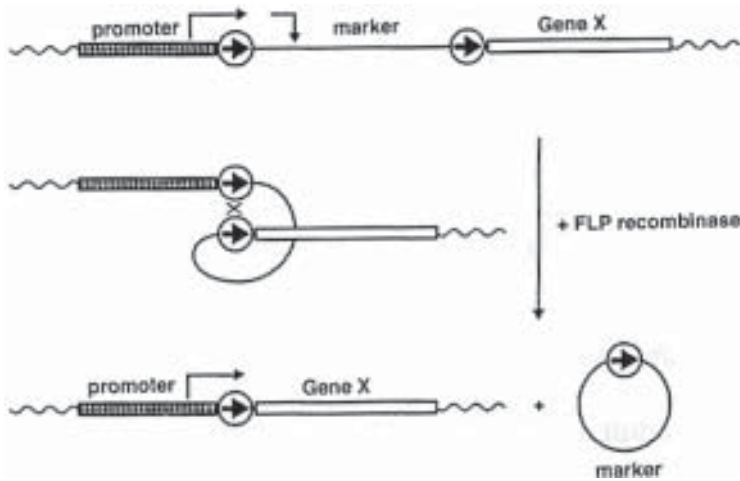
Fig. 2. The Flip-out system of ectopic expression (*see* **ref. 3**). Flip recombinase (FLP) target sites (FRTs) are arranged as direct repeats flanking a visible marker. The expression of this marker is under the control of the promoter element. However, in the presence of FLP, recombination between the FRTs is induced, resulting in deletion of the marker gene. The gene of interest is now juxtaposed to the promoter element, resulting in ectopic expression of the gene of interest. This is an efficient but stochastic process, resulting in clones of cells that express the gene. The area over which the clones are induced is defined by the region in which the promoter is active.

restrictive temperature during embryogenesis and shifted to the permissive temperature at the relevant stages. This permits ectopic activity to begin at the desired stage. However, since temperature sensitive lesions have not been defined for most genes, the inability to control expression temporally remains a problem with the GAL4 system in analysis of postembryonic development.

3.3. Ectopic Expression in Clones

The temporal control of ectopic expression has been critical for the analysis of gene activity during imaginal development. An ingenious method of ectopically expressing genes in any region of the imaginal discs was developed by Struhl and Basler *(3)* (**Fig. 2**) and has come to be called the flip-out system. This method involves the generation of random clones in which the coding region of a gene of interest comes to lie adjacent to a ubiquitous promoter. In these clones, the gene is ectopically expressed, whereas in the surrounding tissue, a gene encoding a visible marker is adjacent to the ubiquitous promoter, separating it from the gene of interest. This is accomplished through the use of flip recombinase target (FRT) sites flanking the marker gene. In the presence of the recombinase, the marker is removed, bringing the promoter and the gene of interest together. The resulting clone of cells is marked by the absence of the marker, which is ubiquitously present elsewhere in the fly.

This technique requires the generation of a construct in which the gene of interest is placed within the context of the promoter-FRT-marker-FRT construct *(3,10,11)*. Two vectors are available that utilize either the Actin-5C promoter or the β-Tubulin pro-

moter. Both of these produce ubiquitous expression, so clones can be generated in any tissue. Levels of expression produced by the Actin-5C promoter are generally higher than those produced by the β-Tubulin promoter. A third vector uses the Ultrabithorax (Ubx) promoter, which produces clones in a more restricted pattern. Transgenic lines carrying the flip-out construct as well as a FLP transgene under the control of the hsp70 promoter (hs-FLP) must be generated. This is done through standard genetic manipulations using any of a number of hsFLP insertions on various chromosomes.

A variation on this method of ectopic expression involves a combination of the GAL4 system and the flip-out system *(12)*. The promoter-driving expression of the FRT cassette, in this instance, is the GAL4 UAS. Clones induced via hs-FLP, therefore, fall only within the domain of GAL4 expression. The advantage of this combination lies in the strength of GAL4 as a transcriptional activator. Clones induced in this way express very high levels of the gene of interest.

The strengths of the flip-out technique are as follows.

1. The clones are efficiently generated randomly throughout the animal. By analyzing a number of animals, it is very likely that clones will be found in a region of interest.
2. Ectopic expression is completely inducible. Lethality because of early expression is avoided.
3. The clones are marked molecularly by the ectopic expression of the gene of interest, and they are marked in the adult cuticle by the absence of the visible marker.

As with any form of clonal analysis, this technique is limited to mitotically active cells, because cell division is required to generate a clone. A second limitation is that randomly generated clones are not reproducible; therefore, clones analyzed in the imaginal discs cannot be analyzed later in the adult cuticle. This contrasts with GAL4-driven expression that generates reproducible phenotypes. In this instance, one can precisely correlate imaginal disc phenotypes with the later phenotypes produced in the adult. Although these limitations need to be considered, the strengths of the flip-out system make it a very useful way to analyze gene activity during imaginal development.

4. Notes

The foregoing approaches provide enormous temporal and spatial control over ectopic expression in *Drosophila*, allowing investigators to analyze gene activity in virtually any cell at any stage of development. However, in addition to the caveats mentioned for each of these methods, a few general concerns should be noted.

1. Positional effects can alter the levels of ectopic expression produced from any transgene. Thus, a transgene under the control of a given regulatory element may not express at the same level as a different transgene under the control of the same element. Therefore, multiple transgenic strains should be generated for any experiment to control for positional effects.
2. Variability in phenotypes produced by ectopic expression is common. The reason for this is apparent with the flip-out system, because clones are randomly generated. Variation can be controlled, however, by inducing the clones within a narrow window of development. By collecting embryos over a short period before aging them and inducing the clones, clone size is kept more constant, as is the timing of ectopic expression relative to other developmental events. Variation in phenotypes using the GAL4 system is less pronounced, but can still be a problem. This can be minimized by rearing flies at a consistent temperature and by maintaining cultures in uncrowded conditions.

References

1. Struhl, G. (1985) Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the Drosophila segmentation gene ftz. *Nature* **318,** 677–680.
2. Brand, A. and Perrimon, N. (1992) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415.
3. Struhl, G. and Basler, K. (1993) Organizing activity of wingless protein in Drosophila. *Cell* **72,** 527–540.
4. Zuker, C. S., Mismer, D., Hardy, R., and Rubin, G. M. (1988) Ectopic expression of a minor Drosophila opsin in the major photoreceptor cell class: distinguishing the role of primary receptor and cellular context. *Cell* **53,** 475–482.
5. Parkhurst, S. M. and Ish-Horowicz, D. (1991) Mis-regulating segmentation gene expression in Drosophila. *Development* **111,** 1121–1135.
6. Fischer, J. A., Giniger, E., Maniatis, T., and Ptashne, M. (1988) GAL4 activates transcription in Drosophila. *Nature* **332,** 853–856.
7. Duffy, J. B., Harrison, D. A., and Perrimon, N. (1998) Identifying loci required for follicular patterning using directed mosaics. *Development* **125,** 2263–2271.
8. Staehling-Hampton, K., Jackson, P. D., Clark, M. J., Brand, A. H., and Hoffmann, F. M. (1994) Specificity of bone morphogenesis protein (BMP) related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A. *Cell Growth Diff.* **5,** 585–593.
9. Wilder, E. L. and Perrimon, N. (1995) Dual functions of wingless in the Drosophila leg imaginal disc. *Development* **121,** 477–488.
10. Diaz-Benjumea, F. J. and Cohen, S. M. (1995) Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121,** 4215–4225.
11. Zecca, M., Basler, K., and Struhl, G. (1995) Sequential organizing activities of engrailed, hedgehog, and decapentaplegic in the Drosophila wing. *Development* **121,** 2265–2278.
12. Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996) Direct and Long Range action of a Dpp morphogen Gradient. *Cell* **85,** 357–368.